analysis at the moment, there is still a need to develop accurate TLC methods for detailed separation of these lipids. Further investigations on the use of new TLC materials in the quantitative analysis of bile components are needed, as well as the adaptation of TLC methods to automated devices. Nevertheless, compared to other analytical tools TLC is the method of choice for fast routine use.

See also: **II/Chromatography: Thin-Layer (Planar):** Densitometry; Layers; Mass Spectrometry; Spray Reagents. **III/Bile Acids:** Liquid Chromatography; Gas Chromatography. **Clinical Diagnosis: Chromatography. Lipids:** Liquid Chromatography; Gas Chromatography; Thin-Layer (Planar) Chromatrography. **Neonatal Metabolic Disorders: Detection: Thin-Layer (Planar) Chromatography**.

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BIOANALYTICAL APPLICATIONS: SOLID-PHASE EXTRACTION

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Introduction

The quantification of drugs and metabolites in biological fluids (e.g. plasma, serum and urine) is one of the most active research fields in clinical and pharmaceutical analysis. Drug bioanalysis is important in clinical chemistry to demonstrate optimal drug therapy, because plasma drug concentrations relate to the therapeutic or toxic effects of a drug. Knowledge of the plasma drug concentration is used to determine why a patient does not respond to drug therapy or why a drug causes an adverse effect. Dosage adjustment by the physician is then warranted. Drug bioanalysis is important in pharmaceutical research to determine the pharmacokinetics and metabolic biotransformation of new drug molecules. It is a technique used throughout the development of all new drugs. In particular, during drug discovery, bioanalysis yields essential data that is used in the decision-making process of whether or not a new molecule should be a candidate for further development.

This chapter discusses the utility of solid-phase extraction (SPE) in comparison with other drug sample preparation techniques for bioanalysis. Several applications of SPE will be summarized in the clinical setting for therapeutic drug monitoring, and in the pharmaceutical research setting for drug discovery and development. The separation and detection techniques used for bioanalysis will be examined, contrasting the use of high-pressure liquid chromatography (HPLC) in the clinical setting and the rapid proliferation of HPLC combined with tandem mass spectrometry (liquid chromatography/mass spectrometry/mass spectrometry or LC/MS/MS) for specific and sensitive detection of drugs for pharmaceutical bioanalysis.

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Drug Sample Preparation Techniques

A reliable analytical method is achieved with efficient sample preparation, adequate chromatographic separation, and a sensitive detection technique. Detailed and exact guidelines exist for the validation of bioanalytical methods, which meet requirements agreed to by the Food and Drug Administration. The sample preparation step is an important component of each overall bioanalytical method, as it

- often concentrates an analyte to improve its limits of detection,
- removes unwanted matrix components that can cause interferences upon analysis, thus improving method specificity, and
- frees the analyte from matrix components so that it can be placed into a solvent suitable for injection into the chromatographic system.

Liquid/liquid extraction

A common sample preparation procedure used to isolate drug analytes from biological matrices is liquid/liquid extraction (LLE). It is quite effective at removing salts and proteins; water-soluble endogenous components often remain in the aqueous phase.

The organic phase containing the extracted analyte is isolated, evaporated to dryness, and reconstituted in liquid chromatographic mobile phase for analysis. One of the benefits of LLE is that with proper selection of solvent and pH, very clean extracts can be obtained with good selectivity for the target analytes. However, the disadvantages of LLE are that

- it is a very labour intensive procedure,
- it requires large volumes of organic solvents which can be expensive to purchase and dispose,
- exposure of personnel to these solvents can be hazardous to health,
- it cannot easily be automated,
- emulsions have been demonstrated to occur,
- evaporative losses can occur upon dry-down with volatile analytes.

Despite its drawbacks, LLE continues to be used for drug bioanalysis when there is adequate labour and its associated costs are not prohibitive, and the sample throughput can be adequately met.

Protein precipitation

A fast and simple method of sample preparation is protein precipitation, also referred to as 'dilute and shoot'. This nonselective technique involves adding a water-miscible organic solvent (e.g. acetonitrile) or inorganic acid (e.g. trichloroacetic acid, 10%) to the biological matrix (usually in a 3 : 1 or 4 : 1 ratio, v/v), centrifuging or filtering to remove precipitated proteins and injecting an aliquot of the diluted supernatant. This technique is often performed in pharmaceutical drug discovery laboratories as the first attempt to prepare samples for bioanalysis. Satisfactory analyses have been demonstrated with this rapid sample preparation approach, but it has disadvantages. This technique dilutes the sample by a factor of four or five, so it is useful only when sample levels are relatively high, typically in the low $\mu g\,mL^{-1}$ range. Also, matrix components are not efficiently removed, and thus may co-elute with the analyte in the isolated supernatant or filtrate. When present, these contaminants have been shown to interfere with detection techniques and lower the signal for the analyte of interest. This approach thus lacks selectivity and problems can arise from column fouling since the precipitation efficiency is not complete.

Solid-phase extraction

Solid-phase extraction has, during the last 18 years, become recognized as a preferred technique for extracting drug analytes from complex biofluids using adsorption chemistry. The attraction of the analyte for a solid phase adsorbent ('sorbent') is exploited to the exclusion of other compounds through a selective wash step. Elution of the analyte is achieved with an organic solvent that disrupts the attraction to the solid sorbent. Solvent exchange is followed by analysis on a chromatographic system. The traditional format for SPE has been single disposable columns and cartridges. Its advantages are that multiple samples can be prepared in parallel, low volumes of solvents are used and procedures can be readily automated. The technology has been improved in recent years with the introduction of more selective solid sorbent chemistries, disc-based SPE devices, smaller bed mass sorbent loading, on-line SPE techniques and introduction of the 96-well plate format for improved productivity.

An on-line SPE technique has recently shown great utility. A commercial device (Prospekt^{TM} (Spark Holland)) combines an autosampler and a solvent delivery unit to aliquot liquid samples into the flow path of solvent. An SPE cartridge is preconditioned and is in-line with the solvent flow. The cartridge retains target analytes while a weak solvent elutes unretained salts and polar matrix components. An optimized sequence of solvents, each with increasing solvent strength, is used to wash out weakly retained components. A final elution with LC mobile phase elutes the analytes of interest from the SPE cartridge and onto an analytical column for chromatographic separation followed by detection.

The autosampler within this device can be preloaded with up to 160 samples and the entire tray can be analysed in an automated procedure. Its advantages are: unattended sample prep and analysis, minimized adsorptive losses, since sample transfers are not performed as in off-line techniques, and trace enrichment of analyte occurs. Some disadvantages are that analysis is serial (although a sample is always being analysed while another is being extracted and prepared for injection) and sample stability may be an issue for some drugs as a result of extended storage times in the autosampler.

Therapeutic Drug Monitoring

Therapeutic drug monitoring (TDM) is an established clinical specialty in which laboratory specialists quantify drug concentrations for the purpose of evaluating therapeutic response. Examples of drugs that are frequently subjected to TDM are antibiotics, antiarrhythmics, antiasthmatics, antidepressants, antiepileptics, and antineoplastics. These drugs possess a narrow range of therapeutic and safe plasma concentration. Therapeutic index (TI) is defined as the ratio between the maximum and minimum plasma concentrations of the drug's therapeutic

range. A narrow range is defined as a ratio of 2 to 3. A TI below 2 infers that the dose that yields a subtherapeutic response is close to the dose that produces toxicity. Most drugs have a TI of greater than 2.

The preferred technique for drug analysis is use of an immunoassay analyser (e.g. fluorescence polarization immunoassay, FPIA) because it is relatively quick, can be automated, and requires minimal technician training for execution. However, newer drugs requiring plasma quantification often do not have an immunoassay developed, so chromatography is almost always used at first. Drugs whose metabolites play a role in their efficacy and clinical interpretation also need to be determined by chromatography, which analyses multiple components simultaneously. Immunoassays are often not selective enough to distinguish between parent drug and metabolite, and interferences can adversely affect results for some drugs. Another difficulty facing clinical laboratories is that more accurate quantitation and detection requirements must be satisfied owing to lower dosages being administered. The proliferation of new drugs also increases the potential for concomitant administration. When the issue of cost is considered, in addition to the drawbacks listed above for immunoassays, chromatographic techniques can become quite attractive. Most often for clinical bioanalysis, detection methods involve ultraviolet or fluorescence detection coupled with liquid chromatographic separation.

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Antidepressants

Tricyclic and newer antidepressant drugs are one of the most frequently monitored classes of therapeutic drugs in the clinical setting. Drug concentrations are monitored in patients for compliance and to ensure that therapeutic blood levels are reached. Also, patients sometimes take multiple antidepressant drugs, often from different physicians, which can be determined from a LC analysis. Immunoassays frequently cross-react with these drugs (e.g., imipramine, amitriptyline, desipramine and fluoxetine) and their metabolites. SPE has been used in some immunoassay kits to measure specifically one tricyclic drug. Overall, LC is advantageous because of its ability to monitor simultaneously multiple drugs and to resolve potential interferences from concomitantly administered drugs.

Solid-phase extraction methods for antidepressant drugs abound in the literature. These drugs are basic and can be adsorbed to reversed-phase sorbents such as C18 and C8 by both reversed-phase attraction and secondary interactions via cationic adsorption to silanols on the silica surface. Polar sorbents such as cyanopropyl, in which the cationic adsorption becomes primary, have also been used successfully. Methods for these drugs typically involve a solvent exchange of organic eluent for aqueous/organic mobile phase. Evaporative losses are always a concern with this step, but can be avoided by use of SPE discs, in which elution is accomplished with a small volume of mobile phase solution.

Corticosteroids

The measurement of steroids (prednisone, cortisone, prednisolone, cortisol, corticosterone, methylprednisolone) in blood is often inaccurate owing to interference from sample matrix and cross-reactivity with chemically similar steroids. For example, antiserum for cortisol is nonspecific and cannot differentiate between cortisol, its metabolites and therapeutically administered steroids. Again, SPE is a preferred technique for drug sample preparation. An efficient method has been reported using C8 sorbent discs, which allows for elution in mobile phase compatible solution for direct injection, eliminating the need for a tedious dry-down and reconstitution step. Steroids are released from proteins by incubation at room temperature with a HCl solution. Neutralization is accomplished by addition of a sodium borate solution. Following centrifugation, the supernatant is loaded onto conditioned C8 discs. A dilute methanol–water solution acts as an efficient wash to remove adsorbed proteins. Elution is performed with acetonitrile, followed by water. The resulting mixture is compatible with mobile phase for direct injection.

Cyclosporin

Cyclosporin, an immunosupressant drug, has many metabolites and is commonly monitored for drug concentrations in the blood of patients who have received an organ transplant. Monoclonal antibody-based immunoassays are in use for this assay, as well as LC methods. Technology for immunoassay detection is constantly being improved, and the tests in use now are reliable. However, LC methods also function quite well, and the issue of antibody versus LC method often rests with cost analysis for a clinic. LC methods rely on SPE using whole blood and, when coupled with automation, can be quite cost effective. An advantage of LC is that it can simultaneously measure several metabolites.

The extraction procedures for cyclosporin are commonly reversed phase. Note that whole blood is preferred to avoid temperature-dependent cyclosporin redistribution. Mixing with acetonitrile–water haemolyses blood, and aliquots of the supernatant are loaded onto conditioned extraction columns. Wash steps may involve a weak concentration of

acetonitrile in water and elution is achieved with methanol or ethanol, or with an alcohol–water solution.

Antiepileptics

Plasma concentrations of antiepileptic drugs are often monitored during therapy, since a therapeutic range has been well defined. These drugs include phenytoin and carbamazepine and their metabolites, phenobarbital and newer agents such as lamotrigine. Solid-phase extraction is commonly performed using reversed-phase sorbents such as C8 and C18. The wash is performed with water, since the more hydrophilic drug lamotrigine is removed from the sorbent bed at low organic concentrations in water. Elution is efficiently accomplished with acetonitrile. Again, the use of the disc SPE formats can allow elution in volumes small enough to eliminate the evaporation step; a small elution volume of acetonitrile is mixed with water and the resulting solution is injected directly onto the liquid chromatograph.

Pharmaceutical Drug Discovery and Development

The discovery and development of new drug entities can be a perplexing task for analytical chemists. Drug molecules that demonstrate activity in receptor assays may exhibit structural characteristics that make them poor candidates for absorption *in vivo*, and other times they may be so rapidly metabolized as to limit their duration of activity in the body. Drugs may be difficult to separate on chromatographic columns, or be so labile that analytical techniques become a challenge. Once these challenges are overcome, the determination of drug concentrations in blood and urine yields the data used to understand the time course of drug action, or pharmacokinetics, in animals and man. Modern requirements for bioanalytical assays include specificity to determine parent drug from metabolites, sensitivity to detect concentrations of ng mL^{-1} and often lower, and speed.

The mainstay for detection following chromatographic separation was formerly ultraviolet or fluorescent detection. These techniques have served the analytical needs well over the years, but newer analytical instrumentation, namely the maturing of MS interfaces to LC, has allowed analytical chemists to use a more powerful detection technique for their routine analyses. The development of more potent drugs has also placed greater emphasis on the determination of lower concentrations of drugs, down to the pg mL^{-1} range. The answer to the analytical need for greater sensitivity, specificity and speed has been LC coupled with tandem mass-spectrometry (LC/MS/MS).

An application illustrating efficient sample preparation required prior to LC/MS/MS analysis is the determination of leukotriene LTE_4 in urine. Leukotrienes are biologically important molecules derived from arachidonic acid by the action of the enzyme 5-lipoxygenase. LTE_4 is difficult to analyse because it is unstable under a variety of conditions. The unique capabilities of MS have yielded detailed structural and metabolic information about these compounds. The extraction of LTE_4 from 5 mL human urine is accomplished by pH adjustment to 4.5 before loading onto a conditioned C18 SPE column or disc. A wash of 5% formic acid is used, followed by elution with a small volume of methanol. The eluate is evaporated and then reconstituted in mobile phase for analysis by LC/MS/MS. This extraction method is simple, yet selective for LTE_4 in human urine. Concentration range tested was 50 pg mL^{-1} to 10 ng mL^{-1}.

A recent advance in improving the throughput of drug development, made possible by LC/MS/MS techniques, is the simultaneous determination of mixtures of drug candidates in single analytical samples as a means to select optimal target drugs. In this case, animals are dosed with several test compounds at once; pooled plasma from multiple animals dosed with single compounds also has been shown. The specificity and sensitivity of the MS detection techniques now available have made this advancement possible. The data generated by this approach have been reported to yield meaningful pharmacokinetic data.

High Throughput Applications of SPE in Bioanalysis

The introduction and utilization of liquid chromatography interfaced with tandem MS techniques has resulted in a dramatic change in sample preparation techniques for drug bioanalysis. The speed of LC/MS/MS, in which run times are typically 1–3 min, allows more samples to be analysed per unit time than traditional HPLC analysis techniques, which require about 10–30 min per sample. The ability of the instrumentation to analyse samples faster than ever before, combined with the emergence of combinatorial chemistry techniques for drug discovery, have put more drugs into the drug development pipeline. The greater number of drugs under evaluation places increased demands on the resources available for pharmacokinetic drug metabolism support. As a result, sample preparation has become the rate-limiting step in achieving higher throughput in bioanalysis.

The pharmaceutical industry has responded to the challenge of higher throughput sample preparation by using a recent advance, SPE in a 96-well microtitre plate format. This technique utilizes single blocks or

plates that have 96 wells containing discs or packed beds of sorbent particles arranged in an 8-row × 12-column rectangular matrix. Although the plates can be processed manually, instrumentation is preferred for processing liquids through the SPE plates. The advent of high throughput sample preparation formats, in combination with the specificity, sensitivity and speed of LC/MS/MS analytical techniques, have created a superior combination for the analytical chemist to meet the demands for faster sample processing and data generation to support the drug development process.

Conclusion

The future of drug bioanalysis using SPE is promising as more and more laboratories increase the usage of LC/MS/MS instrumentation and justify the need for high throughput SPE. LC/MS/MS instrumentation is now common in pharmaceutical laboratories and clinical laboratories are slowly beginning to demonstrate utility for mass spectrometry for certain drug classes, e.g. tacrolimus (FK506, an immunosuppressant). The 96-well plate format is finding its way into many bioanalytical applications; when coupled with automation, there is currently no faster sample preparation method. Individual SPE cartridges will continue to have a role in sample preparation, but 96-well plates will proliferate in pharmaceutical applications. More bioanalytical applications will adopt smaller sorbent mass products, as the productivity gains from reduced solvent volume are realized, especially using automation. As the sorbent mass in plates decreases, the number of applications that demonstrate elimination of the evaporation step by using small elution volumes of mobile phase compatible solution for direct injection will increase.

See also: **II / Extraction:** Solid-Phase Extraction. **III / Drugs and Metabolites:** Liquid Chromatography – Mass Spectrometry. **Solid-Phase Extraction with Discs.**

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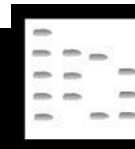
BIOGENIC AMINES: GAS CHROMATOGRAPHY



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Introduction

The term 'biogenic amine' was proposed by Guggenheim in 1940 in order to define the low-molecular-weight organic bases, produced by the decarboxylation of amino acids, that possess biological activity. Biogenic amines are receiving increasing interest because they are often present in foods, such as cheese, meat, and fish where they are used as a useful indicator of spoilage and markers of food quality. They occur naturally in the central nervous system, where they play an important role as neurotransmitters. Their presence in metabolic pathways in health and disease has been studied because of their biological activity as reported by Parvez *et al.*, and they are